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Biodegradation of Paraoxan as an Organophosphate Pesticide with *Pseudomonas plecoglocissida* Transfected by *opd* Gene

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Abstract

Background: Organophosphate pesticides (OP) are applied to agricultural farms and can be carried away into closely sewerage and gullies, which consequently carry water to rivers and lakes and when distributed in the environment they become polluted and require remediation.
Objectives: The current study aimed at producing a genetically engineered *Pseudomonas plecoglossicida* capable of biodegradation of the organophosphate pesticides, paraoxon.
Methods: Genetically engineered *P. plecoglossicida* was initially made by transferring polymerase chain reaction (PCR) product of *opd* gene from *Flavobacterium* sp. ATCC 27551 into the chromosome of *P. plecoglossicida*.
Results: The constructed strain could hydrolyze paraoxon to p-nitrophenol and di-ethylphosphate in paraoxon supplemented in complete supplement mixture (CSM) medium. The isolate could use paraoxon as the only source of carbon. Thus, the bacteria degraded the organophosphate pesticides, and utilized nutrient products of their degradation.
Conclusions: The observed versatility of genetically engineered *P. plecoglossicida* in biodegradation of xenobiotics suggested that this strain may be useful for the multipurpose bioremediation of contaminated agricultural and industrial sites.

Keywords: Organophosphates, Pesticide, Bioremediation, *Pseudomonas plecoglossicida*

1. Background

Tremendous development of chemical industry in the past century made possible the present quality of life, but as a side effect, enormous amounts of synthetic chemicals are discharged into the environment either intentionally (as waste deposits, fertilizers, or pesticides) or accidentally. Therefore, today a remarkable number of earth-bound and marine habitats are contaminated by various chemical compounds which many of them are harmful for living organisms.

Due to their impact and threat for human health, organophosphorus compounds are of great concern. Organophosphate pesticides are a heterogeneous group with a phosphoric acid derivative chemical structure. These synthetic chemicals have a cholinesterase-inhibiting activity and at the present time, organophosphate pesticides are widely used in the developing countries (1). Occupational exposure in agricultural industries and self-poisoning with organophosphate pesticides produce remarkable health problems (2-7). Exposure to organophosphate pesticides results in millions of poisoning (8-11). Organophosphate pesticides are utilized directly to the soil and can be washed off into the nearby sewerage and gullies, and consequently carry water to rivers and lakes and when these substances distributed in the environment they become polluted and require remediation (12-14). Degradation of organophosphate compounds by microbial enzymes is the focus of biodegradation researches. Organophosphate-degrading bacteria have the same organophosphate hydrolyzing enzymes. OP hydrolase is a zinc-containing protein found to hydrolyze paraoxon (15). The enzyme is encoded by an organophosphate degradation gene (*opd*) and can hydrolyze a variety of oxon and thion organophosphates (16, 17). The *opd* is reported in different taxonomic groups of soil microorganisms, which suggested its spread by transposons, plasmids, and phages as transfer vehicles (18, 19).

Previous studies suggested *Pseudomonas* species as competent microorganisms for microbial remediation and degradation (14, 20, 21) and within this genus, *Pseudomonas plecoglossicida*, which is a soil habitant, attracted much attentions (22).

2. Objectives

The current experimental study aimed at constructing new genetically engineered *P. plecoglossicida* capable of degradation of paraoxon as an organophosphorus pesticide.

3. Methods

Chemicals, enzymes, and oligonucleotides: All chemical reagents including paraoxon (O,O-diethyl O-p-nitrophenyl phosphate) were obtained from Sigma-Aldrich (Tehran, Iran). Proteinase K purchased from Roche (Tehran, Iran), and synthetic dNTPs from Takapou Zist (Tehran, Iran).

Bacterial growth: Carbon-deficient medium (CSM) had the following composition: 0.2 g/L $MgSO_4.7H_2O$; 0.08 g/L $Ca(NO_3)_2.4H_2O$; 0.005 g/L $FeSO_4.7H_2O$; 4.8 g/L KH_2PO_4; 1.2 g/L KH_2PO_4. CSM was supplemented with an appropriate carbon source just before inoculation. Luria-Bertani medium (LB) with the following composition was used: 1.5 g/L Bacto agar; 10 5 g/L yeast extract; g/L Bacto tryptone; 10 g/L NaCl; all purchased from Difco Laboratories, Detroit, MI, USA. The pH of both mediums was 7.5.

Evaluation of biodegradation effect of modified *P. plecoglossicida* on paraoxon: To assess the biodegradation effect of modified *P. plecoglossicida* on paraoxon, the bacterial suspension was spread on CSM agar plates containing increasing concentrations of organophosphate. Paraoxon concentrations in agar were 100, 200, 300, and 400 μg/mL. The plates were incubated at 37°C for 20 hours (23).

Degradation of organophosphate by resting cells: Degradation experiment of OP was performed as previously described by Lyer et al. (23). Briefly, 150 mL of LB medium was inoculated with transfected *P. plecoglossicida* on a rotary shaker at 37°C with 200 rpm rotation until cell density reached 1.0 at OD = 600. Cells were harvested, washed, and re-suspended in the same potassium phosphate buffer. The degradation experiment was done by mixing the cell suspension with 100 μg/mL of paraoxon and incubation at 30°C and 120 rpm of shaking. At regular intervals aliquots were taken, centrifuged, and analyzed to remove the test compound and accumulation of its degradation products. Decomposition of organophosphate pesticides was monitored by high performance liquid chromatography under the previously described conditions (24). Briefly, the study used 25 $\times$ 14 mm C18 Hichrom column with 80% acetonitrile, 19.5% distilled deionized water (DDW) and 0.5% acetic acid solution as mobile phase; flow rate was 1.5 mL/minute and detection was done at absorbance 246 nm. All tests were repeated 3 times. All statistical tests were conducted by SPSS ver.14 software.

3.1. Isolation of Cellular DNA

Cetyl trimethylammonium bromide (CTAB) method was employed for total cellular DNA extraction as described before. Briefly, cells were resuspended in TE buffer, containing 50,000 Units/mL lysozyme and 300 K Units/mL ribonuclease A, and maintained for 1 hour at 37°C. Prior to lysis, 0.25 mg/mL of proteinase K was added and cells were lysed by application of 0.5% (w/v) sodium dodecyl sulfate (SDS) at 37°C for 1 hour and DNA was precipitated by standard phenol chloroform procedure.

3.2. Amplification and Insertion of Organophosphorus Hydrolase Gene Into P. plecoglossicida

Amplification of OP hydrolase gene was evaluated by polymerase chain reaction (PCR) with the following primers from parathion hydrolase gene of *Flavobacterium* sp. ATCC 27551; forward primer: CGCCACTTTCGATGCGAT; reverse primer: CTTCTAGACCAATCGCACTG (23). PCR program was as follows: 4.5 minutes at 94°C, 32 cycles at 94°C for 30 seconds, 55°C for 30seconds and 72°C for1 minute with a final elongation step at 72°C for 4 minutes. PCR products were analyzed by electrophoresis in a 0.8% agarose gel in Tris/borate/EDTA (TBE) buffer. Separated DNA fragments were excised from the gel, purified as described, and then, 1700-bp *opd* genes (25) were inserted into the pUC57 plasmid according to the instruction of the Template Generation System II kit (Thermo Scientific, Nedaye Fan, Tehran, Iran), and transfected to *P. plecoglossicida*. Briefly, 4 μL of amplified *opd* gene DNA; 10 μL of H_2O; 4 μL of 5X reaction buffer; 1 μL of MuA transposase, and 1 μL of Entreanceposon™ (CamR-3) were mixed and incubated for 1 hour at 30°C; then, incubated at 75°C for 10 minutes and after that, the amplified plasmid was transformed into Stella competent cells. Presence of the plasmid in *P. plecoglossicida* was confirmed by amplification by the following PCR mixture: 14.4 μL of H2O; 4 μL of 5X Phire reaction buffer; 0.4 μL of 200 μM each dNTPs (10 mM each); 0.4 μL of each pUC forward and reverse primers (25 μM) 0.5 μM; 0.4 μl of Mu end primer (25 μM) 0.5 μM; 0.4 μL of Phire Hot Start II DNA Polymerase, and 20 μL of the following primer sequences:

Mu end primer: 5'-GTTTTTCGTGCGCCGCTTCA-3'; pUC reverse primer: 5'-TTATGCTTCCGGCTCGTATGTTGTGT-3' or pUC forward primer: 5'-AGCTGGCGAAAGGGGGATGTG-3' according to the following program: 98°C for 30 seconds, 30 cycle of 98°C for 5 seconds, and 72°C for 1 minute. A 1750-bp PCR final product indicated the presence of plasmid.

4. Results and Discussion

The bacterial strain designated as modified *P. plecoglossicida* was created by inserting PCR product of the amplification of *opd* gene of *Flavobacterium* sp. ATCC 27551 into

the pUC57 plasmid, and transfection of *P. plecoglossicida* with this plasmid. Pure culturing of transfected strain in paraoxon supplemented CSM medium changes the color in the culture medium to yellow, which confirmed its ability to hydrolyze paraoxon to p-nitrophenol and diethylphosphate. The isolated bacteria grew on carbon-deficient agar containing 100 to 400 μg/mL paraoxon as available carbon sources (Figure 1). This result suggested that the strain could degrade the organophosphate pesticides and use the products of this biodegradation.

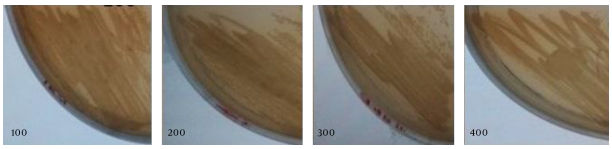

Figure 1. Growth of Modified *Pseudomonas plecoglossicida* on CSM Agar Supplemented with 100 - 400 μg/mL Paraoxon as the Only Available Carbon Sources

Degradation of paraoxon by modified P.plecoglossicida was started with a 100 μg/mL of paraoxon. Concentration of p-nitrophenol, as a byproduct of degradation of paraoxon in the media was evaluated by absorbance at 405 nm. The effect of spontaneous OP hydrolysis was corrected by subtracting the amount of p-nitrophenol formed in the presence of Escherichia coli ATTC 25922/pUC57 cells as non-organophosphate pesticides degrading control (data not shown).

Detection of the gene in the transfected *P. plecoglossicida* was performed by analytical PCR with *opd* specific primers (23). PCR, on the whole, modified *P. plecoglossicida* genomic DNA, which resulted in a single amplicon for each of the F450 - R840 primers (Figure 2).

Chromatograms of paraoxon after 24 hours of inoculation in CSM broth containing 400 μg/mL of paraoxon showed that the concentrations of paraoxon decreased after incubation with *P. plecoglossicida* suspension. These chromatograms showed a reverse relationship between incubation time and concentration of paraoxon in the culture tubes (Figure 3).

Organophosphate pesticides are directly applied to the agricultural farms and can be washed off into the nearby sewerage and gullies, which are consequently carried by water to rivers and lakes and when spread in the environment they become polluted and require remediation (12-14).

The ability of transfected *P. plecoglossicida* to proliferate in the environment containing toxic substances by using these substances as nutrients suggests tight control over their uptake and intracellular concentration. The discovered biodegradation capabilities of transfected

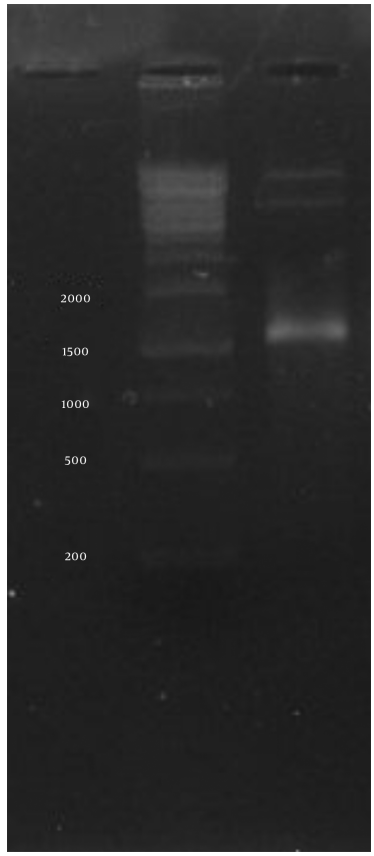

Figure 2. Detection of *opd* Gene in the Transfected *Pseudomonas plecoglossicida* by Analytical PCR Using *opd* Specific Primers

P. plecoglossicida may find application in a variety of tasks. The strain can be used to accelerate internalization of organophosphate pesticides in agricultural systems, sewage water, and leaks of petroleum hydrocarbons and related industrial chemicals.

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Footnotes

Authors' Contribution: Taghi Naserpour Farivar: designing the study, performing the experiment and data analysis; Masoumeh Aslani Mehr and Reza Najafipour: data analysis; Pouran Johari: performing the experiments and data

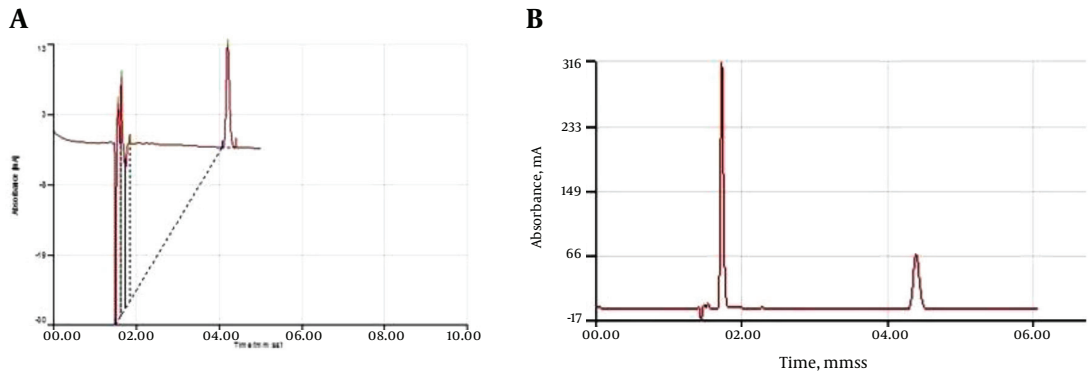

Figure 3. A, Paraoxon (400 μg/mL) in Carbon-Deficient Minimal Medium; B, After 24 Hours Of Incubation with 0.5 mL of Modified *Pseudomonas plecoglossicida* Suspension (0.5 McFarland); 25 × 14 mm C18 Hichrom Column Was Used with 80% Acetonitrile, 19.5% DDW, and 0.5% Acetic Acid Solution as Mobile Phase; Flow Rate was 1.5 mL/min and Detection Was Done at the Wave Length of 246 nm. All tests were repeated 3 times.

analysis; Amir Peymani and Safar Ali Alizadeh: designing the study, performing the experiment and data analysis

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